WHEY PROTEIN FRACTIONATION BASED ON Q-SEPHAROSE ANION EXCHANGE CHROMATOGRAPHY

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A thesis submitted in fulfillment
of the requirements for the award of the Degree of
Bachelor of Chemical Engineering

Faculty of Chemical & Natural Resources Engineering
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DECEMBER 2010

ABSTRACT

Whey proteinsarewell known for their nutritional value and had versatile functional properties.The mains proteins present in whey are α-lactalbumin, β-lactoglobulin, immunoglobulin andbovin eserum albumin. In this study, whey protein component is separate into several fractions using Q-Sepharose anion exchange chromatography column. The chromatographic process was run at different pH range from pH5 to pH8. The best separation was achieved at pH6 in which the major protein component can be separated into their individual protein fraction especially contains α-lactalbumin and β-lactoglobulin. The yield of α-lactalbumin and β-lactoglobulin recovered from 2 ml whey at pH 6 was 46% and 98.4% respectively. Fractionation of single and pure protein component from whey can add the value of the wheybased product and these proteins can be used for specific application.

ABSTRAK

Whey protein sangat terkenal disebabkan oleh nilai nutrisinya dan memiliki sifat fungsional serbaguna. Komponen utama protein yang hadi rdalam whey adalah seperti α-lactalbumin, β-lactoglobulin, imunoglobulindan albumin bovine serum. Dalam kajian ini, komponen whey protein diasingkan menjadi beberapa bahagian protein menggunakan Q-Sepharose kromatografi medan penukar anion. Proses kromatografi dijalankan pada julat pH yang berbeza mulai dari pH 5 ke pH 8. Pengasingan komponen whey protein yang terbaik adalah pada pH 6 di mana komponen utama protein dapat dipisahkan menjadi beberapa bahagian protein,terutama yang mengandungi α-lactalbumin dan β-lactoglobulin. Peratusan yield bagi α-lactalbumin dan β-lactoglobulin, dari 2 ml whey pada pH 6 adalah masing–masing 46% dan 98.4%. Pengasingan komponen protein tunggal dan asli dari whey dapat menambah nilai produk berasaskan whey dan ini boleh digunakan untuk aplikasi tertentu.

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α	-Alpha
β	-Beta

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Whey is the greenish translucent liquid that separates from clotted milk during the manufacture of cheese or industrial casein. Whey proteins have a high nutritional value and excellent functional properties (Cayot and Lorient, 1998). It contains many protein components such as α-lactalbumin, β-lactoglobulin, immunoglobulin, bovine serum albumin, lactoferrin, lactoperoxidase and glycomacropeptide (Doultani *et al*., 2003). Whey proteins are well known for their nutritional value and versatile functional properties that are widely used in the food industry (Alomirah *et al*., 2003).Whey is now generally regarded as a functional food, which has measurable effects on health outcomes, and the bioactive properties of whey proteins and whey protein fractions are becoming increasingly recognized (Kruger *et al*.,2005).The demand of pure protein products has been increasing rapidly which triggers the research of more efficient technologies for protein separation (Cheng *et al*., 2010).

Numerous isolation methods have been proposed for separation of major whey protein components. These processes fall into three main categories which are selective precipitation induced by adjustment of the solution physical properties, membrane filtration based primarily on differences in size and charge and selective adsorption (Doultani *et al*., 2003).Ion-exchange chromatography is a selective adsorption process that allows the separation of molecules based on their charge. It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids. The benefits of ion exchange chromatography including high throughput, the

column has a long lifespan and it is a well-known method. For whey proteins separation and whey protein concentrates production, ion exchange chromatography had been developed and utilized successfully on a commercial scale (Gerberding *et al*.,1998).In this study, ion exchange chromatography will be use to fractionate whey protein into single protein component.

1.2 Problem Statement

Whey normally regard as a byproduct in cheese manufacturing. It normally converts into whey based product for bulk usage such as for animal food stock or in confectionary industry. However, this whey-based product had a low selling price. One way to increase the value of whey products is by isolating specific protein from whey. Beside high value price, it can be used for specific application. However, the whey protein fractionation process very challenging and need a detail study for it successful. Chromatography is among the success technique for this purpose and had advantages over others techniques.

1.3 Research Objective

The main objective of this study is to fractionate whey protein components into several group or single protein fraction by using Q-Sepharose anion exchange chromatography column operate at different whey pH.

1.4 Research Scope

In order to fulfill the research objective, the following scopes were outlined:

1. Prepare and analysis the whey protein composition from fresh milk.
2. Setup and operated anion exchanger chromatography using AKTA Explorer 100 liquid chromatography system.
3. Study the fractionation of whey protein at different pH from pH 5to pH8.
4. Characterize and analysis the protein fraction using reverse phase chromatography (RPC).

CHAPTER 2

LITERATURE REVIEW

2.1 Whey Protein

Milk contains two primary proteins which are casein and whey protein. Whey denotes the greenish translucent liquid that separates from clotted milk during manufacture of cheese or industrial casein. Casein can be precipitated by acidification to pH 4.5-4.8 or through the action of rennet, a casein-coagulating enzyme. Figure 2.1 shows the major component present in cow milk. Protein only represents about 2-6% of total milk component. Casein is the major protein in milk and the rest of the protein is categorized as whey protein.

2.1.1 Whey Protein Component

Whey protein contains different type of protein that varies in its concentration and properties as showed in Table 2.1.The major proteins present in whey include α-lactalbumin, β-lactoglobulin, immunoglobulin, bovine serum albumin (BSA), and glycomacropeptide. Minor proteins are lactoferrin (LF) and lactoperoxidase (LP) (Doultani *et al*., 2003).

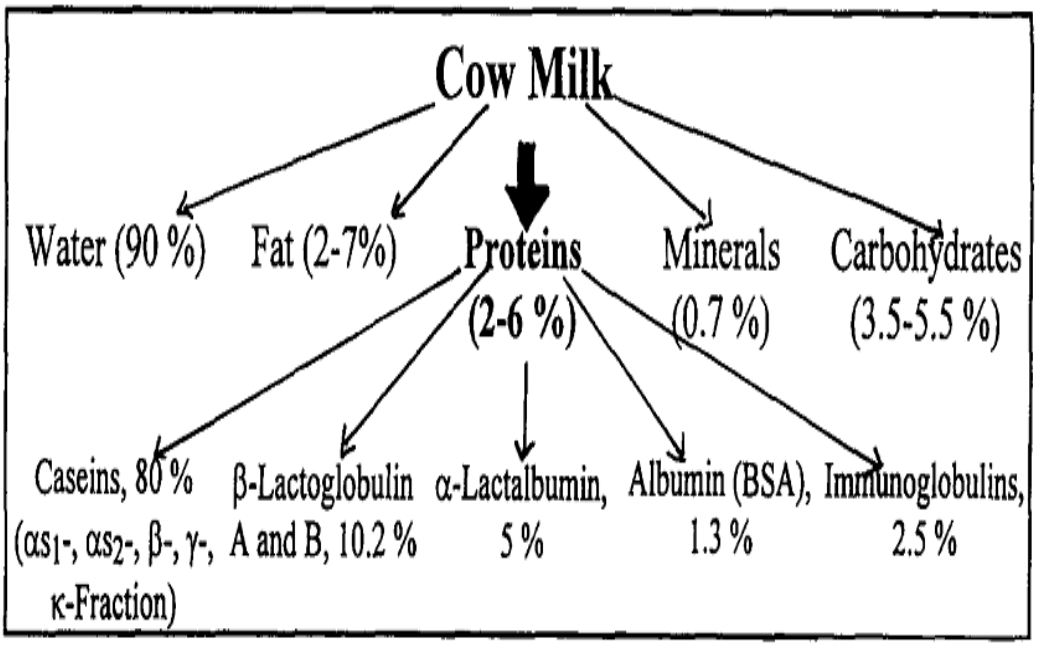

Figure 2.1: Major components founds in bovine milk (Splitt *et al.,* 1996).

Table 2.1: Protein composition of bovine whey (Andersson and Mattiasson, 2006)

Protein	Concentration in Whey (g/l)	Molecular Weight (KDa)	Isoelectric Point
β-lactoglobulin	2-4	18	5.2
α-lactalbumin	1.2-1.5	14	4.5-4.8
Immunoglobulins	0.6-0.9	150-1000	5.5-8.3
BSA	0.3-0.6	69	4.7-4.9
Lactoferrin	0.02-0.2	78-92	8-9.5
Lactoperoxidase	0.02-0.05	78-89	9.5
Glycomacropeptide	1-1.2	7	<3.8

2.1.3 Application of Whey

The most important functional properties of whey proteins are solubility, viscosity, water holding capacity, gelation, emulsification and foaming. These properties had made it widely being used in food industries. Table 2.2 summarizes whey protein properties that are useable in various types of products.In addition to their general properties, individual whey proteins have their own unique nutritional, functional and biological characteristics (Almecija *et al.*, 2006).

Table 2.2: Whey protein properties that are useable in various types of products (Walsh, 2001).

Products Category	**Example of Product**	**Desired Properties**
Bakery products	Bread,cakes,muffins,croissants	Nutritional,emulsifier,egg replacer
Dairy products	Yoghurt,quarg,ricotta cheese	Yield, nutritional, consistency,curd cohesiveness
	Cream cheese, cream cheese spread,cheese fillings and dips.	Emulsifier, gelling, sensory properties
Beverages	Soft drink,fruit juices,powdered or frozen orange juices	Nutritional
	Milk-based flavored beverages	Viscosity, colloidal stability
Dessert products	Ice-cream, frozen dessert	Whipping properties,body and texture.
Confectionary	Aerated candy mixes,meringues, sponge cakes	Whipping properties, emulsifier
Meat products	Frankfurters, luncheon meats	Pre-emulsion, gelation
	Injection brine for fortification of whole meat products	Gelation

2.2 Protein Separation Technique

Several techniques can be used for fractionation of individual whey protein. These include salting out, selective precipitation, trichloroacetic acid precipitation, heating at low pH, affinity chromatography, anion exchange chromatography, cation exchange chromatography using conventional resins or cation membranes, size exclusion chromatography, hydrophobic chromatography and combination of enzymatic treatment and membrane filtration (Alomirah *et al.,* 2003). The most common technique is a chromatographic based separation.

2.2.1 Precipitation

Precipitation is an important traditional method for purifying proteins and nucleic acids. According to Vogt.,*et al.(*1997), precipitation is an established, well-understood and easily scaleable operation in preparative protein chemistry. The most common example of precipitation based on bioseparation is the Cohn fractionation method for purifying plasma proteins. Using this method, which consists of an array of precipitation steps, the various component proteins of human plasma are obtained in pure forms (Ghosh, 2006).

Crystallization is one of the precipitation processes where the solid is obtained in form of crystalline. Solubility of compounds is more soluble in hot liquids than they are in cold liquids. If a saturated hot solution is allowed to cool, the solute is no longer soluble in the solvent and forms crystals of pure compound. Precipitation of α-lactalbumin (α-lac) by whey can be achieved by adjusting the physical properties of whey to promote insolubility α-lac from whey by using heat, acid or ultrahigh pressure (Outinen *et al.,*Bramaud *et al.*, Hinrichs and Alomirah and Alli, 2003). Impurities are excluded from the growing crystals and the pure solid crystals can be separated from the dissolved impurities by filtration.

2.2.2 Membrane

Membrane is a thin semi-permeable barrier that separate component based on the size different. Membrane can be made from organic polymers or inorganic material such as glass, metals and ceramics or even liquids (Ghosh, 2006).Membranes are effective in separating the protein mixtures for the protein size differ at least a factor of 10(Van Reis *et al.*, 1996).Membrane separation is commonly used in many applications such as for product concentration, product sterilization (i.e. removal of bacteria and virus particles), solute fractionation, and solute removal from solution, purification and clarification.

2.2.3 Chromatography

Chromatography is a solute fractionation technique which relies on the dynamic distribution of molecules to be separated between two phases: a stationary (binding) phase and a mobile (carrier) phase (Ghosh, 2006).The stationary phase (i.e. chromatography media or particles) is packed within a column in the form of a packed bed. The mobile phase is passed through the column, typically at a fixed velocity. A pulse of sample containing the molecules to be separated is injected into the column with the mobile phase. The velocities at which these molecules move through the column depends on their respective interactions with the stationary phase. For instance, if the velocity does not interact with the stationary phase its velocity is almost the same as that of the mobile phase. With molecules that do interact with the stationary phase, the greater the extent of interaction, the slower is the velocity (Ghosh, 2006).

Chromatography is used for the separation of different substances such as protein, nucleic acids, lipids, antibiotics, hormones, sugars and etc. Some of the applications of chromatography are in biopharmaceutical production, biopharmaceutical and biomedical analysis, environmental analysis, foods and nutraceutical production, diagnostics and process monitoring (Ghosh, 2006).

2.2.3.1 Ion Exchange Chromatography

Ion exchange chromatography is one of the most valuable types of chromatography that had been widely used for protein separation (Wheelwright, 1991). There are two types of ion exchanger column available which are anion exchanger and cation exchanger. Example of anion exchanger column is Q-Sepharose and for cation exchanger is SP-Sepharose. Ion exchange chromatography separate proteins based on different in their electric charge. Among the advantages of ion exchange chromatography technique are it can give very high resolving power, high capacity (capable of large scale purification) and the process is relatively easy to control.

Proteins are made up of amino acids that contain various chemical groups attached to a peptide backbone. These groups may be positively or negatively charged, or they may be electrically neutral. The ion exchange resin contains electrically charged functional group that is covalently attached onto the resin matrix. When the protein is introduced into ion exchange column, it will be attracted to the opposite charges of the column, while the same charges protein will not be bound. The bound protein can be recovered from the ion exchange column by increase the ionic strength of the buffer (ionic strength gradient) used or by changing the pH (pH gradient) of the system to manipulate the charged of the bound protein (Sadana, 1998). Figure 2.2 show example of elution step that use ionic strength gradient protocol. Several protein peaks are recovered depending on the strength of interaction between the bound proteins with the ion exchanger group on the column.

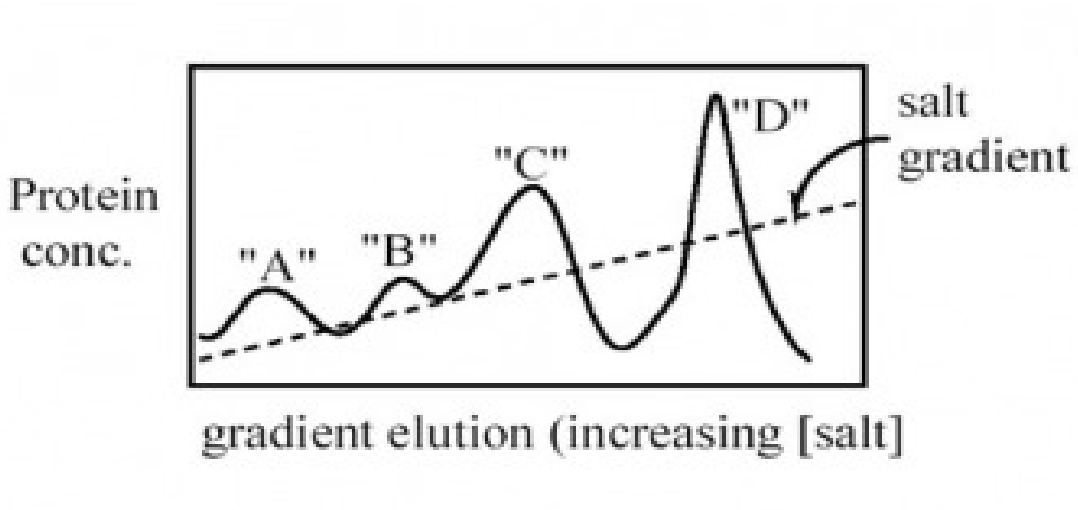

Figure 2.2: Ionic strength gradient applied during the elution step

CHAPTER 3

METHODOLOGY

3.1 Experimental Works

In order to separate whey protein components, the experimental works are divided into three major sections as showed in Figure 3.1. The first step involve on the preparation of whey protein, running buffer and elution buffer. Next step is setup and run the whey protein fractionation experiment with anion exchange chromatography (AEC). The final step is analyzing the fraction obtained from AEC experiment using reverse phase chromatography (RPC).

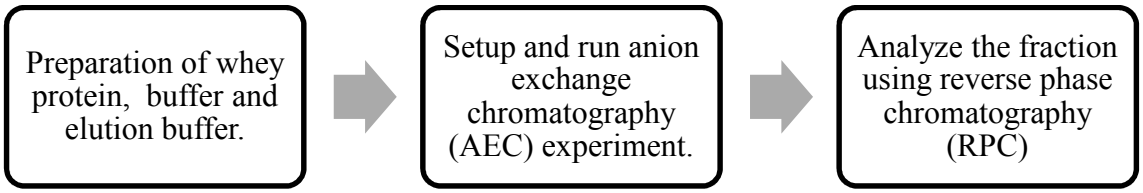

Figure 3.1: The experimental work of separating whey protein components.

3.2 Preparation of Whey

The fresh milk used in experimentation was obtained from First Dairy Farm (M) Sdn. Bhd in Muadzam, Kuantan, Pahang. Whey was prepared according to the method described by Hahn et al. (1998). Milk was centrifuged in an Eppendorf refrigerated centrifuge at 6049.7 rpm at room temperature for 30min for delipidation. The Ph of the skimmed milk was adjusted to 4.7 by the slow addition of 1 M HCl. After casein precipitation, the solution was stirred for a further 30min to complete precipitation. Casein was removed by centrifugation at 12106 rpm and 4°C for 30min. The pH was readjusted to the desired pH of the study with HCl or NaOH. Before use in chromatography experiment, the whey was filtered through a 0.45 µm membrane filter. Figure 3.2 summarized all the steps involve in whey preparation used in this study.

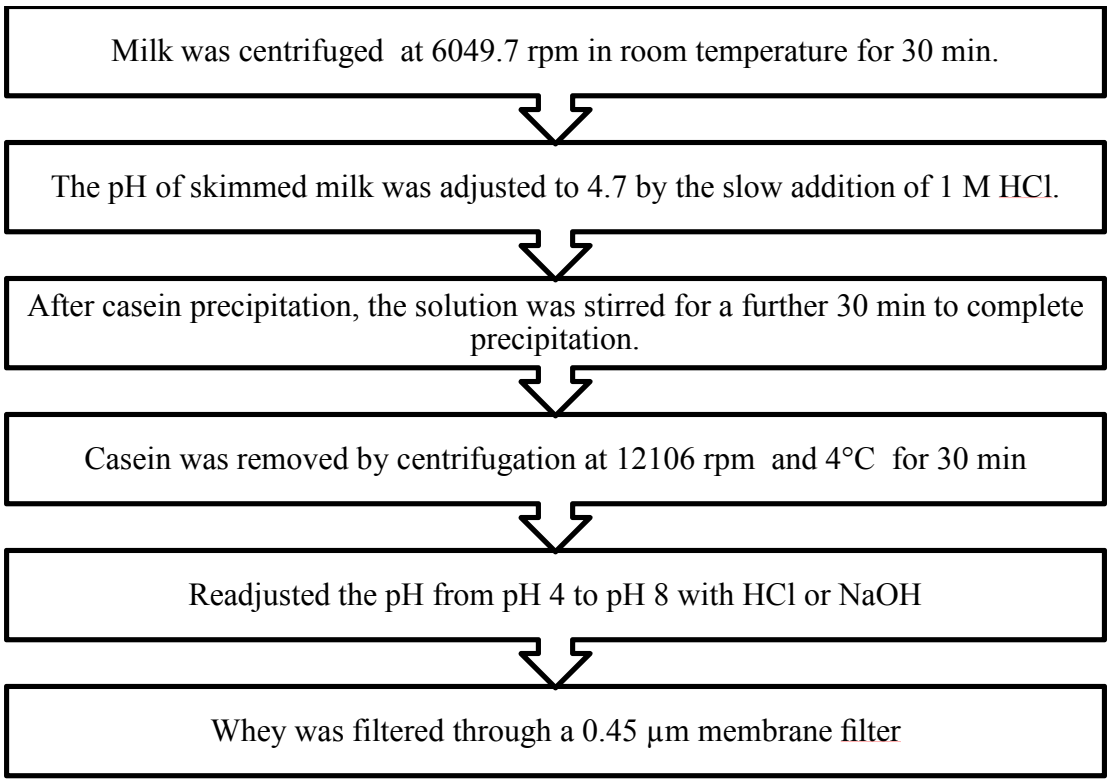

Figure 3.2: Preparation of whey from fresh milk.

3.5 Preparation of Buffer Solution

Table 3.1 shows the buffer and cleaning solution that used in this study. Ion exchanger chromatography experiment will be run at different pH; therefore different type of binding buffer was prepared. Washing buffer is used as same as binding buffer. For elution buffer, 1 M NaCl will be added to the binding buffer to recover the bound protein from ion exchange column.

Table 3.1: Preparation of buffer solution and cleaning solution

<table>
<tr><td rowspan="2">pH</td><td colspan="3">Buffer Solution</td></tr>
<tr><td>Binding Buffer</td><td>Elution Buffer</td><td>Cleaning Buffer</td></tr>
<tr><td>4</td><td rowspan="2">Acetate Buffer</td><td rowspan="2">Acetate Buffer + 1M NaCl</td><td rowspan="5">0.5M Sodium Hyroxide</td></tr>
<tr><td>5</td></tr>
<tr><td>6</td><td rowspan="3">Phosphate Buffer</td><td rowspan="3">Phosphate Buffer + 1M NaCl</td></tr>
<tr><td>7</td></tr>
<tr><td>8</td></tr>
</table>

3.6 Anion Exchange Chromatography

Q-Sepharose anion exchange resin was packed in Tricon column for the chromatographic fractionation of whey. The column was showed in Figure 3.3.Two steps was involved on packing the column which are media preparation and column packing.

Q-Sepharose media was rinsed three times with deionized water in vacuum filter apparatus. After rinsing, the media was dissolved in deionized water to achieved 50% volume concentration.

The packing was done in AKTA Explorer liquid chromatographic system. Two empty columns were connected in series by packing connector adapter. The media was

poured into the column and top column adapter was connected to the AKTA system. The media was packed by running the pump at certain flow rate until no movement of media was detected. Then, the column was detached from the AKTA and the top column was removed. The bottom column was fixed with new top adapter and was tested for column performance test before can be used in chromatographic experiment.

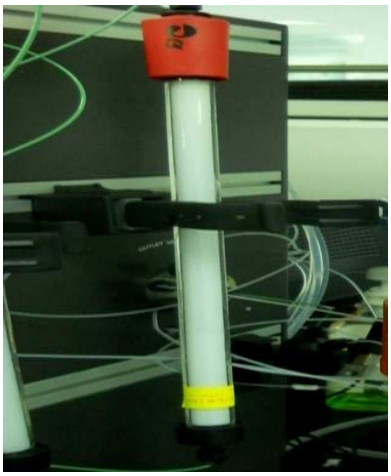

Figure 3.4: Q-Sepharose Column Anion Exchange Chromatography

3.6.1 Protein Analysis by Reverse Phase Chromatography

The protocol similar to Elgar et al., (2000) was used for reverse phase chromatography (RPC) analysis. 1ml Resource RPC column was operated at room temperature and at a flowrate of 2 ml/min. The column was equilibrated in 80% solvent (0.1%,v/v,TFA in Milli-Q water) and after sample injection a 1-miniisocratic period was applied followed by a series of linear gradients to 100% solvent B (0.09%,v/v,TFA,90%,v/v, MeCN in Milli-Q water) as follows:1–6min,20–40%B;6–16min,40–45%B;16–19min,45–50%B;19–20min,50%B;20–23min,50–70%B;23–24min, 70–100%B. The column was re-equilibrated after a 1-min hold at 100% B by a 2min

linear gradient to 20% B followed by an isocratic period of 3min. Detection was by absorbance a 214nm and total run time was 20min.